NY-ESO-1-specific immunological pressure and escape in a patient with metastatic melanoma

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Abstract: During cancer progression, malignant cells may evade immunosurveillance. However, evidence for immunological escape in humans is scarce. We report here the clinical course of a melanoma patient whose initial tumor was positive for the antigens NY-ESO-1, MAGE-C1, and Melan-A. Upon immunization with a recombinant vaccinia/fowlpox NY-ESO-1 construct, the patient experienced a mixed clinical response and spreading of the NY-ESO-1 epitopes in the CD4+ T cell compartment. After NY-ESO-1 protein + CpG immunization, the patient's anti-NY-ESO-1 IgG response increased. Over the following years, progressing lesions were resected and found to be NY-ESO-1-negative while being positive for MAGE-C1, Melan-A, and MHC-I. The fatal, inoperable brain metastasis was analyzed after his death and also proved to be NY-ESO-1-negative, while being positive for MAGE-C1 and Melan-A, as well as MHC-I. We propose that cancer control and cancer escape in this patient were governed by NY-ESO-1-specific immunological pressure. Our findings provide evidence for the existence of immunoediting and immunoescape in this cancer patient.

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NY-ESO-1–specific immunological pressure and escape in a patient with metastatic melanoma

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During cancer progression, malignant cells may evade immunosurveillance. However, evidence for immunological escape in humans is scarce. We report here the clinical course of a melanoma patient whose initial tumor was positive for the antigens NY-ESO-1, MAGE-C1, and Melan-A. Upon immunization with a recombinant vaccinia/fowlpox NY-ESO-1 construct, the patient experienced a mixed clinical response and spreading of the NY-ESO-1 epitopes in the CD4+ T cell compartment. After NY-ESO-1 protein + CpG immunization, the patient's anti-NY-ESO-1 IgG response increased. Over the following years, progressing lesions were resected and found to be NY-ESO-1-negative while being positive for MAGE-C1, Melan-A, and MHC-I. The fatal, inoperable brain metastasis was analyzed after his death and also proved to be NY-ESO-1-negative, while being positive for MAGE-C1 and Melan-A, as well as MHC-I. We propose that cancer control and cancer escape in this patient were governed by NY-ESO-1-specific immunological pressure. Our findings provide evidence for the existence of immunoediting and immunoescape in this cancer patient.

Keywords: NY-ESO-1, Cancer/Testis antigen, melanoma, immunosurveillance, escape

Introduction

The immune system can control cancer cells through a process known as cancer immunosurveillance. However, tumors evolve with time such that they escape immune control (editing). Most evidence for immune-driven editing and escape was generated in mouse models (1, 2) and proof for the existence of such processes in humans is scarce. Tumor-specific peptides arise from mutated or uniquely expressed proteins in malignant cells, such as from Cancer/Testis (CT) antigens like NY-ESO-1 and MAGE-C1 (3). Because spontaneous responses to CT antigens are found in cancer patients, these antigens are thought to be immunogenic and therefore represent attractive targets for cancer immunotherapy (4-5). Accordingly, a variety of different NY-ESO-1 vaccines have been investigated in clinical trials (6-12).

Here, we present an immunological characterization of a melanoma patient who lived almost 10 years with metastatic disease. The patient was treated with various forms of surgery, chemo-, radio-, and immunotherapy. Importantly, he was immunized with vaccinia/fowlpox NY-ESO-1, followed by NY-ESO-1 protein + CpG. We found that all lesions growing out in the face of immunization were negative for NY-ESO-1, while being positive for other antigens like MAGE-C1, Melan-A, and MHC-I. Our findings provide evidence for the existence of immunoediting and -escape in this cancer patient.

Results

Treatment history

A 61-year-old male patient (ZH-311) was diagnosed in March 2001 with an inguinal lymph node metastasis from an occult primary melanoma. The clinical course of ZH-311 is remarkable, as he lived for more than 9 years with metastatic Stage IV melanoma. The treatment history is summarized in Figure 1. The initial tumor mass was widely excised and adjuvant interferon alfa-2a therapy was given. A year and a half after the initial diagnosis, the patient developed multiple mesenterial lymph node and liver metastases. A combination therapy with interferon alfa-2b and temozolomide was started and initially stabilized the disease. Upon recurrent tumor growth, vindesine treatment was initiated but had to be stopped early due to neuropathy. The tumor typed positive for NY-ESO-1 at diagnosis and the patient fulfilled the inclusion criteria for the LUD 00-014 clinical trial, exploring immunization efficiency with a recombinant vaccinia/fowlpox NY-ESO-1 construct (7). The immunization was initiated in May 2004 when the patient presented with liver, lymph node, and peritoneal metastases. In August 2004, he came to the emergency room with bleeding *ab ano* caused by a metastasis in the colon. This metastasis was excised and the immunization was continued until a single brain metastasis was discovered in March 2005. This metastasis also was excised, followed by local irradiation. All other tumor sites at that time were regressing. In a follow-up scan in May 2005, a new pulmonary metastasis was diagnosed but, as requested by the patient, no specific treatment was initiated. In September 2005, the patient was offered a NY-ESO-1 protein + CpG immunization after informed consent. In

Figure 1

Date	Treatment
Mar 2001	First diagnosis inguinal lymph node metastasis
Jun 2001	Adjuvant Interferon α2a
Feb 2003	Chemotherapy with Interferon α2a and Temozolomid
Mar 2004	Chemotherapy with Vindesine
Mar 2004	Vaccinia/Fowlpox anti NY-ESO-1 vaccination
Aug 2004	Colon metastasis surgery
Apr 2005	Brain metastasis surgery and radiotherapy
Sep 2005	Anti NY-ESO-1 protein + CpG vaccination
Mar 2006	Lung metastasis surgery
Sep 2006	Liver metastasis surgery
Jul 2007	Ipilimumab
Apr 2008	Abdominal wall metastasis surgery
Mar 2009	Stop Ipilimumab
Jul 2010	Death due to inoperable brain metastasis

Clinical course and treatment history of patient ZH-311.

March 2006, the then still growing lung metastasis was excised. No other metastases except for stable liver metastasis were detected by imaging and, in September 2006, it was decided to remove the remaining tumor in the liver. In June 2007, he developed a new abdominal wall metastasis upon which treatment with ipilimumab was initiated in July 2007 and continued until March 2009. An initial mild colitis resolved rapidly upon steroid treatment. The abdominal wall metastasis was surgically removed in April 2008 due to continuous growth. The patient died in July 2010 from a new inoperable brain metastasis more than 9 years after the initial diagnosis of metastatic melanoma.

In summary, besides the initial immuno-chemotherapy and adjuvant brain irradiation, the patient was treated with two NY-ESO-1-specific immunizations, ipilimumab and five surgical interventions for metastatic disease to the colon, brain, lung, liver, and the abdominal wall. Four surgeries were performed to control single progressing metastases and the surgery of the stable liver metastasis was performed in hope to render the patient tumor-free (Figure 1).

Longitudinal analysis of tumor antigen and MHC-I expression in metastases

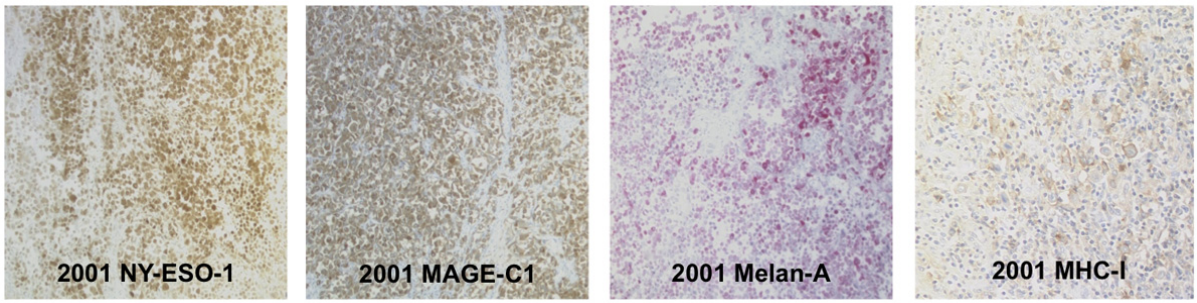
The inguinal lymph node metastasis, which was excised at initial diagnosis in March 2001, expressed the CT antigens NY-ESO-1 and MAGE-C1, as well as Melan-A and MHC-I (Figure 2). Tumor sites, which were removed after immunization due to progressive disease (colon, brain, lung, and abdominal wall metastases), did not express NY-ESO-1 (Figure 3), but the sites were MAGE-C1- and Melan-A-positive (Figure 4). In September 2006, the stable liver metastasis was removed. In contrast to the progressing metastases, the stable liver metastasis expressed NY-ESO-1 (Figure 3). The fatal brain metastasis, which was analyzed post mortem had the same expression profile as all the progressing tumor lesions (NY-ESO-1-negative). Otherwise, post mortem tumor was found in the lungs and pulmonary lymph nodes; these tumor sites were NY-ESO-1-negative. The liver was free of disease. All tumors expressed MHC-I molecules as assessed by immunohistochemistry. More thorough analysis of MHC-I alleles was not performed (13).

In conclusion, examination of NY-ESO-1 expression in different lesions revealed that progressing lesions after immunization were NY-ESO-1-negative, whereas the stable lesion still expressed NY-ESO-1. The expression of MHC-I, MAGE-C1, and Melan-A remained unchanged during the course of the disease. We propose that NY-ESO-1-specific immunity prevented the outgrowth of NY-ESO-1-positive tumor cells but did not impact on NY-ESO-1-negative tumor cells.

NY-ESO-1-specific CD8+ T cell responses and antibodies against NY-ESO-1

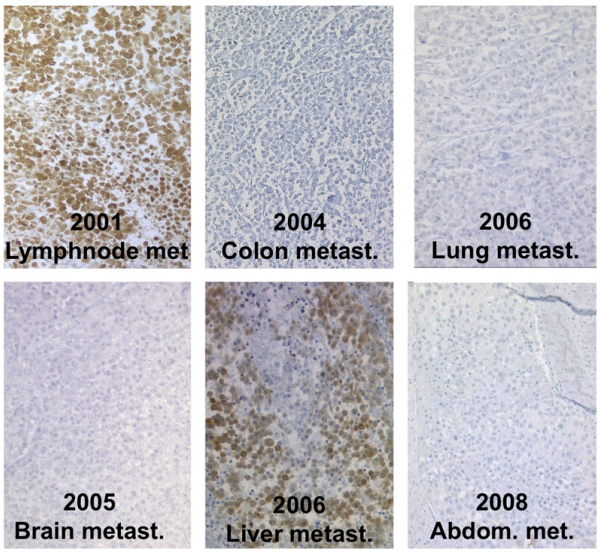
The patient was continuously lymphopenic (< 1000 lymphocytes/μl), a condition that is often observed in cancer patients. CD8+ T cell responses against NY-ESO-1 were investigated by staining for cytokine production (IFN-γ and TNF-α) and degranulation (CD107a surface expression) after *in vitro* stimulation. Initially, overlapping peptides spanning the entire NY-ESO-1 sequence were used and we found that the majority of the CD8+ T cells were specific for two peptides (Figure 5): aa 71-90 (peptide 3) and aa 81-100 (peptide 4), confirming the results obtained during the LUD 00-014 clinical trial. All further experiments were thus performed with the combination of these two peptides. A polyfunctional CD8+ T cell response against NY-ESO-1 was observed in peripheral blood mononuclear cells (PBMCs) before immunotherapy (patient 31 in (7)), which seemed to increase during vaccinia/fowlpox immunization (Figure 6B and Figure 7). However, analyses were performed after 3 weeks of *in vitro* expansion, making the assays less quantitative. After NY-ESO-1 protein + CpG immunization, only the capacity to degranulate increased, whereas the percentage of IFN-γ and TNF-α producing CD8+ T

Figure 2



Immunohistochemical analysis of the initial tumor biopsy. Expression of NY-ESO-1, MAGE-C1, Melan-A, and MHC-I in the initial tumor in March 2001.

Figure 3



Immunohistochemical analysis of NY-ESO-1 expression in different biopsies. Expression of NY-ESO-1 was present in the initial tumor in March 2001 and in the stable liver metastasis in 2006. All other tumor sites were negative for NY-ESO-1.

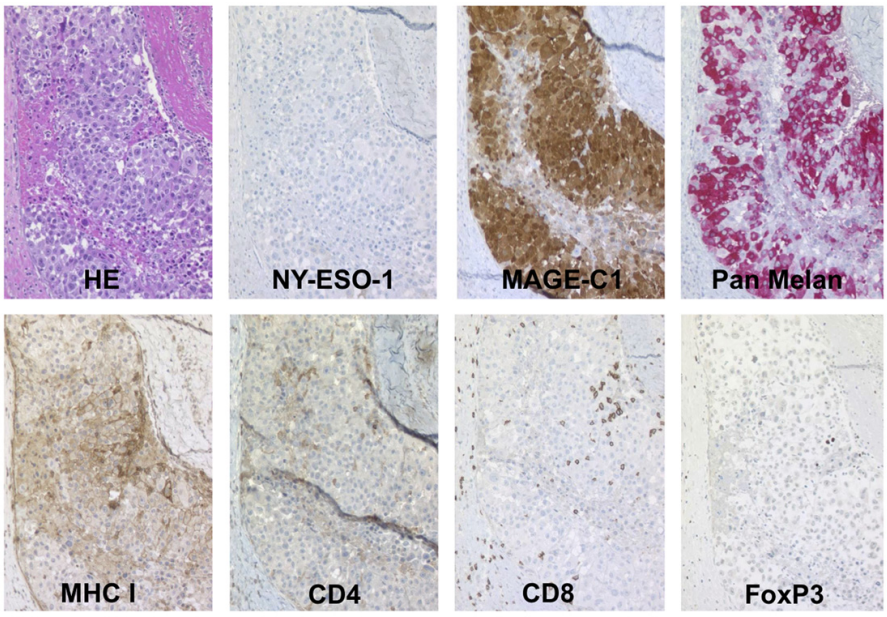
cells did not change. The cellular response was analyzed 3 weeks after *in vitro* stimulation and was mirrored by the NY-ESO-1-specific IgG response initially (Figure 6A); however, the NY-ESO-1-specific IgG remained high, whereas the NY-ESO-1-specific CD8+ T cell response decreased and could not be boosted or recalled by immunization with NY-ESO-1 protein + CpG or ipilimumab (Figure 6C).

Discussion

The adaptive immune system evolved to control and eliminate non-self. There is ample evidence that it is able to recognize malignant cells as well: immunodeficient mice develop more carcinogen-induced cancers than wild-type mice (1, 14-17), tumor cells from immunodeficient mice are more immunogenic than those from immunocompetent mice (14), and the immune system of a naïve mouse can restrain cancer growth for extended time periods (18). Evidence for the existence of such processes in humans is scarce and, due to obvious reasons, indirect. Spontaneous tumor-specific immune responses are frequently detected in cancer patients (19-20) and, along this line, we detected an anti-NY-ESO-1 immune response in the patient described here. However, it is challenging to prove a causal relation between the existence of such responses and tumor control or escape (15-17). There are reports that correlate the quantity, quality, and spatial distribution of tumor-infiltrating lymphocytes (TILs) with patient survival or patient outcome under therapy. Remarkably, the type and density of lymphocytes infiltrating lung (23) or colon cancer (24) was found to be a more powerful prognostic indicator than previous pathological criteria for tumor staging. Recently, a strong association between immune cell density of colorectal liver metastases and chemotherapy response was described, highlighting the importance of the local immune response in metastatic colorectal cancer (CRC) (25).

Immunodeficiency in organ-transplanted or in AIDS patients is associated with a higher risk of cancer. Although the cancers arising in these patients are typically those with a viral etiology such as lymphomas (Epstein-Barr virus), Kaposi's sarcoma (herpes viruses), and cervical cancer (human papilloma viruses), there is at least some evidence that these patients are at greater risk for malignancies of the colon, lung, pancreas, kidney, head and neck, and endocrine system, as well as nonmelanoma skin cancers (26, 27).

Figure 4

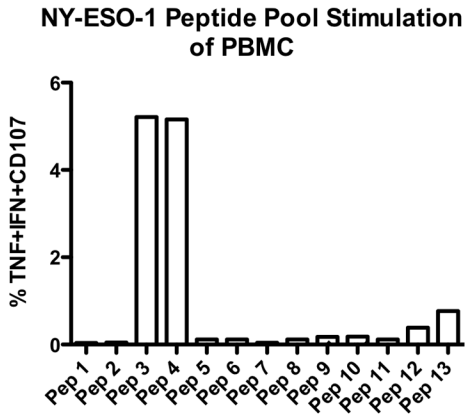


Immunohistochemical analysis of the abdominal wall metastasis. Expression of NY-ESO-1, MAGE-C1, Melan-A, and MHC-I in a metastasis that was surgically removed in April 2008.

Patient ZH-311 had a spontaneous NY-ESO-1-specific humoral and cellular immune response, which was boosted by subsequent immunizations. Despite this, some lesions grew progressively whereas others were stable or decreased. We interpret the observation that NY-ESO-1 expression was lost selectively in progressively growing lesions as a clear indication for immune escape. We thus propose that persistence and progression of NY-ESO-1-negative cancer cells was driven by NY-ESO-1-specific immunological pressure. As the patient harbored other targetable tumor antigens, a multivalent vaccine (including MAGE-C1) might have prevented the outgrowth of NY-ESO-1-negative tumors.

In summary, these clinical observations and laboratory findings are consistent with the hypothesis that malignancies are immunoedited not only when the unmanipulated immune system encounters a developing tumor, but also when an established tumor is treated with immunotherapy.

Figure 5



Spontaneous NY-ESO-1-specific immunity in patient ZH-311. Intracellular staining for IFN-γ and TNF-α and for degranulation (CD107a surface expression) after *in vitro* stimulation of ZH-311 PBMCs. Overlapping peptides spanning the entire NY-ESO-1 sequence were used for stimulation and readout. aa 71-90 correspond to peptide 3 and aa 81-100 correspond to peptide 4.

Abbreviations

CT, Cancer/Testis; PBMC, peripheral blood mononuclear cell; TIL, tumor-infiltrating lymphocyte; APC, antigen-presenting cell

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Materials and methods

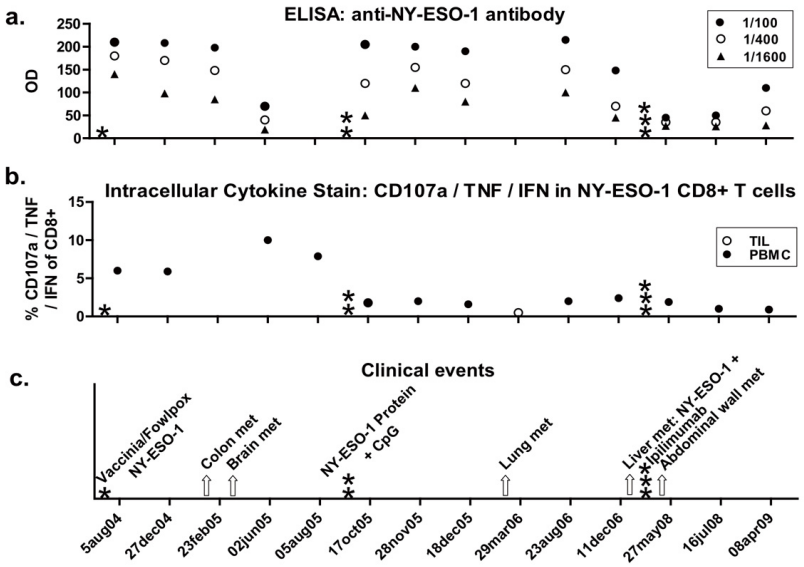
Patient

A 61-year-old male patient (ZH-311) was diagnosed with metastatic melanoma with an unknown primary lesion upon discovery of an inguinal mass in March 2001. The clinical course and treatment details are given in Figure 1. The patient gave informed consent for analyses described in accordance with the Declaration of Helsinki.

Measurement of NY-ESO-1-specific CD8+ T cell responses

To characterize NY-ESO-1-specific CD8+ T cell responses in PBMCs and TILs during the course of disease, we performed *in vitro* stimulation with NY-ESO-1-derived overlapping peptides (20-mers) followed by intracellular cytokine staining. CD8+ and CD4+ T cells were isolated from PBMCs by positive selection using the MACS system (Miltenyi Biotech, Germany) according to manufacturer's instructions. CD4+ and CD8+ fractions were > 90% pure as determined by flow cytometry (data not shown). The remaining CD8- CD4- fraction was irradiated (20 Gy), loaded with 10^{-6} M of each of two overlapping NY-ESO-1 peptides (aa 71-90 and aa 81-100) and was used as antigen-presenting cells (APCs). $4x10^5$ CD8+ T cells were incubated for 8 days with $4x10^5$ peptide-loaded, irradiated APCs in 96-well flat bottom plates in 200 µL RPMI containing 10% heat-inactiv-

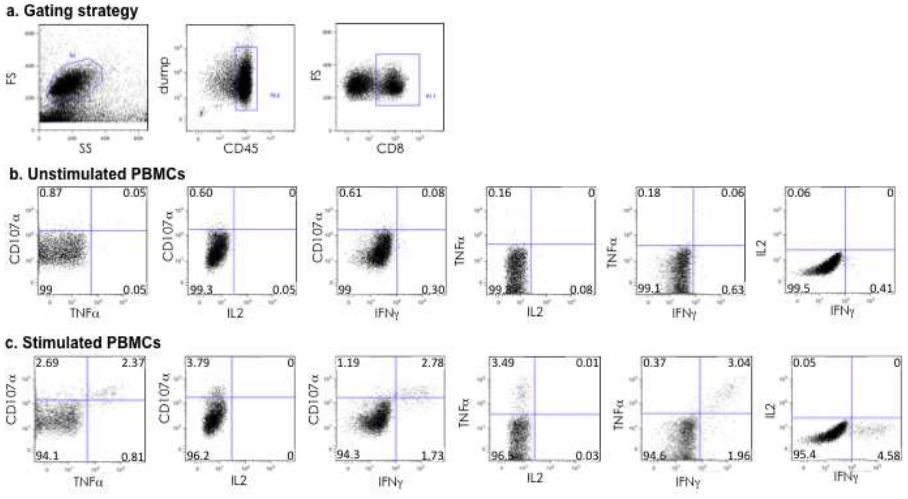
Figure 6



Immunological parameters and clinical events. (A) NY-ESO-1-specific IgG. (B) NY-ESO-1-specific polyfunctional CD8+ T cell response. (C) Clinical events. Immunization with vaccinia/fowlpox NY-ESO-1 is indicated with (*), immunization with NY-ESO-1 + CpG with (**), and ipilumumab with (***) on the X-axes. Arrows indicate the time points of operation.

ated human serum, antibiotics, and 100 U/mL IL-2. During the same period, PHA-blasts (T-APC) were generated from CD4+ T cells: 10^6 CD4+ T cells per mL RPMI containing 10% heat-inactivated human serum, antibiotics, and 100 U/mL IL-2 in 24-well plates were activated with 1 μg/mL phytohaemagglutinin (PHA). After 8 days, T-APC were harvested and labeled with 1 μM CFSE. The CD8+ T cell cultures were resuspended and incubated for 6 hours with CFSE-labeled T-APC +/- peptide (10^{-6} M) in the presence of 10 μg/mL Brefeldin A, 10 μg/mL monensin, 1 μg/mL anti-CD28/anti-CD49d antibodies (BD Biosciences), and PE-labeled anti-CD107a antibodies. After incubation, cells were harvested and stained with anti-CD3-Pacific Orange, anti-CD4-APC-Cy7, anti-CD8-PE-Texas Red, anti-CD14-Pacific Blue, anti-CD19-Pacific Blue, and violet LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen). After fixing (2% formalin) and permeabilization (0.1% saponin in PBS), cells were stained intracellularly with anti-IFN-γ-APC and anti-TNF-α-PE-Cy7. All antibodies were obtained from BioLegend or Beckman Coulter (anti-CD8-PE-Texas Red). The percentage CD107a+, IFN-γ+, TNF-α+, and CD8 cells was determined after gating on CD3+ CD8+ cells and excluding dead cells (Violet-), monocytes, and B cells (CD14- and CD19-, respectively).

Figure 7



NY-ESO-1-specific immunity in patient ZH-311. (A) Representative flow cytometry dot plots for gating strategy. Representative flow cytometry dot plot of intracellular staining for IFN-γ, TNF-α, and for degranulation (CD107a surface expression) unstimulated (B) and after *in vitro* stimulation (C) of ZH-311 PBMCs.

Measurement of NY-ESO-1-specific serum IgG

We tested sera for the presence of NY-ESO-1-specific IgG with GST-tagged protein by ELISA. The dilutions were performed for each time point. ELISA was performed as described earlier (28).

Immunohistochemical analysis of the expression of NY-ESO-1, MHC-I, and immune infiltration of tumor samples

Tumor specimens from surgery and all tumor sites revealed in autopsy were analyzed by immunohistochemistry for the expression of NY-ESO-1 and MHC-I, as well as for CD4+ and CD8+ T cell infiltration. NY-ESO-1 immunohistochemistry was performed as recently reported (29, 30). In brief, paraffin-embedded tissue sections were stained with mouse anti-human monoclonal antibodies (CD4, clone 1F6, 1:30, Zymed Laboratories, Inc.; CD8, clone C8/114B, 1:100, Dako A/S, Carpinteria, CA; MHC-I, clone 2l, 1:1000, RDI Research Diagnostics, Inc., this antibody does not detect all alleles; NY-ESO-1, clone E978, 1:50, Zymed Laboratories, Inc.). UView (Ventana) or Refine DAB (Vision BioSystems) were used as chromogens against the primary antibodies. Sections were counterstained with hematoxylin, dehydrated, and mounted. All sections were stained with the Ventana BenchMark automated staining system (Ventana Medical Systems) using Ventana reagents for the entire procedure.

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